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Characterization of Tick-Borne Enchephalitis Virus From Latvia

Violeta Mavtchoutko,[1] Sirkka Vene,[2,3] Mats Haglund,[4,5] Marianne Forsgren,[5] Arnis Duks,[1] Vaira Kalnina,[1] Jan Hörling,[3,6] and Åke Lundkvist[2,3]*

[1]*National Environmental Health Centre, Riga, Latvia*
[2]*Swedish Institute for Infectious Disease Control, Solna, Sweden*
[3]*Microbiology and Tumour Biology Centre, Karolinska Institute, Stockholm, Sweden*
[4]*Department of Infectious Diseases, Huddinge University Hospital, Huddinge, Sweden*
[5]*Division of Clinical Virology, Huddinge University Hospital, Karolinska Institute, Huddinge, Sweden*
[6]*Pharmacia & Upjohn AB, Stockholm, Sweden*

Viruses of the tick-borne encephalitis (TBE) antigenic complex, within the family *Flaviviridae*, cause a variety of diseases including uncomplicated febrile illness, encephalitis, meningoencephalitis, hemorrhagic fever and chronic disease in humans, domesticated animals or wildlife species. TBE is a serious problem in Latvia with up to a 1,000 patients confirmed serologically annually 1994–1995. No previous data had been reported on the causative agent of TBE in Latvia. In the present study, a virus was isolated from serum of a patient with clinical symptoms of an acute TBE infection. Nucleotide sequence information obtained by direct reverse transcription-polymerase chain reaction (RT-PCR) and the serological characteristics of the isolated virus strain, designated TBE-Latvia-1-96, indicated a closer relationship to the Vasilchenko strain, isolated in Novosibirsk (Siberia, Russia), as compared to the western European or far eastern subtypes of TBE viruses. In a mouse neurovirulence assay, a significant difference in survival rates (days) was shown between Latvia-1-96 and the western European TBE virus subtype. ***J. Med. Virol. 60:216–222, 2000.***

KEY WORDS: tick-borne encephalitis virus; flavivirus; ticks; zoonosis

INTRODUCTION

Tick-borne encephalitis virus (TBEV) is one of the major human pathogenic viruses among the flaviviruses, together with dengue, yellow fever and Japanese encephalitis viruses. The viruses are enveloped particles containing a single positive stranded RNA genome approximately 11 kb in length, encoding three structural proteins, the capsid (C), the membrane (M) and the envelope (E) proteins and seven non-structural proteins [Rice, 1996].

Two subtypes of TBEV, a western and a far eastern subtype have been identified which are transmitted by the ticks, *Ixodes ricinus* and *I. persulcatus*, respectively [Monath and Heinz, 1996]. Strains of TBE virus from far eastern Asia (FETBE) cause severe encephalitis with extensive involvement of the central nervous system and a reported case fatality rate of up to 20% [Monath and Heinz, 1996]. Western European strains of TBE (WTBE) are antigenically very closely related to FETBE but produce a less severe clinical picture [Kunz, 1992]. The virus is endemic in many countries of central and eastern Europe, as well as in Asia.

The disease caused by WTBE is typically biphasic and is characterized by a viremic phase presenting with fever, headache and myalgia generally 1–2 weeks after infection. After a latency phase of about one week, 25% of the patients develop clinical signs of central nervous system (CNS) involvement (meningitis, encephalitis, encephalomyelitis or polyradiculitis). Sequelae are observed in approximately 35% of the patients and 1–2% of the cases are fatal [Kunz, 1992; Haglund et al., 1996; Günther et al., 1997]. TBE is diagnosed by the detection of virus-specific IgM in serum and/or cerebrospinal fluid [Hofmann et al., 1990; Günther et al., 1997].

The TBEV complex further consists of viruses related antigenically sharing a high degree of amino acid similarity (77–98%) in the E protein [Mandl et al., 1993] and producing a wide variety of clinical syndromes. Louping ill (LI) virus is found in the UK, Ireland, and Norway causing fatal encephalitis in sheep, grouse and only rarely affects humans [Reid, 1988]. Langat (LGT) virus from Malaysia [Smith, 1956] and

*Correspondence to: Åke Lundkvist, Swedish Institute for Infectious Disease Control SE-171 82 Solna, Sweden.
E-mail: Åke.Lundkvist@smi.ki.se

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the Vasilchenko (Vs) strain of TBE virus from Siberia [Asher, 1979; Frolova et al., 1982] have been reported as attenuated forms of TBE virus, whereas Omsk hemorrhagic fever (OHF) virus from Siberia [Lvov, 1988] produces predominantly hemorrhagic disease. On the basis of the correlation between the geographical and genetic distances, Zanotto et al. [1995] suggested a cline of TBE complex viruses across the northern hemisphere.

The molecular mechanisms defining the different pathogenic features of the flaviviruses have not been clarified. The tropism of virus for secondary target organs might be limited at any stage of virus reproduction; therefore a mutation in any virus protein could be responsible for the altered virus phenotype and pathogenicity. The functions of the E-protein are the best studied to date. This protein mediates targeting of flaviviruses to vital organs by binding to cell receptors. The E protein also induces neutralizing, hemagglutination-inhibiting and protective antibodies [Heinz et al., 1981].

This report describes the isolation of a virus from a Latvian patient with symptoms of TBE, the results of virological and serological characterization of the isolate as well as partial sequence analysis of the E protein gene.

MATERIALS AND METHODS

TBE Virus Strains

TBE strains Sofjyn, the far eastern prototype [Pletnev et al., 1990] and Hochosterwitz, similar to the Neudoerfl western prototype strain [Heinz and Kunz, 1981], were used in the study. Virus strains were propagated in Vero cells cultivated in Eagle's minimal essential medium (MEM) supplemented with 2% fetal calf serum (FCS), 2 mM L-glutamine, 60 μg/ml penicillin and 100 μg/ml streptomycin.

Sera

Sera were collected from patients with acute clinical symptoms consistent with the first phase of TBE (fever, headache) when admitted to the Central Hospitals of Aluksne and Liepaja regions in Latvia, and were stored at −70°C until used. To avoid inactivation of the virus by antibodies, samples from the viremic phase were selected for virus isolation on the basis of nondetectable TBE-specific IgM activity. A second serum sample, obtained 10–26 days after onset of disease, was analyzed for confirmation of the TBE diagnosis by demonstration of TBE-specific IgM.

Antiserum to TBE virus was obtained by immunization of rabbits with inactivated TBE-vaccine (FSME-Immun™ Inject; Immuno AG, Vienna, Austria). Rabbits were given five doses of 0.25 ml of the vaccine at one month intervals, and serum was drawn six months after the first immunization. Hamster and bank vole *(Clethrionomys glareolus)* antisera were produced by s.c. inoculation of infectious virus (strains Latvia-1-96, Sofjyn and Hochosterwitz). Antisera were collected 4–5 weeks after virus inoculation.

Virus Isolation

Initial virus isolation was carried out in suckling white mice. Two-day-old mice were inoculated intracerebrally with approximately 0.01 ml of patient serum, and the animals were examined daily for up to 14 days. Brain suspensions of sick mice were assayed by immunoblotting for the presence of TBEV E antigen. For passage in Vero cells (ATCC no CCL81) cultivated in Eagle's minimal essential medium (MEM) supplemented with 2% fetal calf serum (FCS), 2 mM L-glutamine and antibiotics, 0.05 ml of TBEV E antigen positive brain suspensions were inoculated in 25 cm^2 flasks and incubated at 37°C for four days. Cell suspensions were passaged to fresh monolayers of Vero cells and incubated for further three days. Virus stocks were stored at −70°C until used.

Antigen Detection

Immunoblotting. For immunoblotting, proteins were separated by a standard sodium-dodecyl sulfate polyacrylamide gel electrophoresis (4–15% SDS-PAGE) and transferred to nitrocellulose filters. Filters were preabsorbed with 2% bovine serum albumin in phosphate buffered saline (PBS), incubated over night at 4°C with a cocktail of nine anti-TBEV E protein-specific mouse monoclonal antibodies (clones 19/75, 171, 324, 694, 367, 1418, 1493, 1718, 1786; [Niedrig et al., 1994]), followed by alkaline-phosphatase-labelled conjugates (Sigma, St. Louis, MO) for 2 h. Specific antibody binding was detected with 0.6 mM 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Sigma) and 0.5 mM nitro-blue tetrazolium (NBT, Sigma) in 0.2 M Tris/HCL, 10 mM $MgCl_2$, pH 9.5.

Immunofluorescence Assay. Virus infected Vero cells were examined by immunofluorescence assay (IFA) essentially as previously described [Lundkvist et al., 1991]. Briefly, TBEV strains were incubated for 3 days in Eagles MEM supplemented with 2% FCS, 2mM L-glutamine, and antibiotics, before applying the cells to microscope slides. Mouse Mabs, animal and late convalescent human sera were serially diluted in PBS and incubated for 1 hr at 37°C. After washing, FITC-conjugated rabbit anti-mouse IgG (Dako a/s, Glostrup, Denmark), goat anti-human IgG (Sigma) or goat anti-rabbit (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) immunoglobulins were incubated for 1 hr at 37°C.

Antibody Detection

IgM ELISA. Patients were confirmed serologically as acute TBEV infection by a μ-capture IgM enzyme-linked immunosorbent assay (ELISA) with peroxidase labelled antigen, essentially as previously described [Hofmann et al., 1990].

Neutralization Test (RFFIT). A fluorescent focus inhibition test for detection of neutralizing antibodies was employed as previously described [Vene et al., 1998]. Briefly, serum dilutions were mixed with virus at approximately 50 FFD_{50} (50% focus forming dose) in

96-well tissue culture microplates. Plates were incubated for 90 min at +37°C and 5% CO_2 and a BHK-21-S13-cell suspension was subsequently added to wells. Following a 24 hr incubation, the plates were acetone-fixed and air-dried. Foci were visualized by staining with rabbit-anti-TBEV serum and appropriate conjugate (cf. IF-assay). Twenty microscopic fields/well were examined for fluorescent foci and the number of positive fields with one or more focus were recorded. Neutralizing antibody titers were calculated as the reciprocal of the serum dilution that reduced the challenge-virus to one FFD_{50}.

PCR Amplification and Nucleotide Sequencing

RNA from homogenized, virus infected mouse-brain suspensions were extracted by the acid guanidinium thiocyanate-phenol-chloroform method [Chomczynski and Sacchi, 1987]. The RNA was denatured by incubation at 95°C for 2 min, and subsequently quenched on ice, before reverse transcription and polymerase chain reaction (PCR).

The 5′end of the E-gene (nucleotide positions 885 to 1310) was amplified using a pair of degenerated primers; TBEA (T(A/G)C CGT TGT GTG G(C/T)T GAC CCT GGA) and TBE2 (AAT GCT (C/A)CC (T/C)TT TCC AAA (T/C)A. The PCR was performed at the following conditions; 50 pmol of each primer, 5 U of Rous-associated virus 2 reverse transcriptase (Amersham International, Bucks, UK), 10 U of placental ribonuclease inhibitor, and 2 U Taq polymerase, in 50 μl of PCR buffer (Perkin Elmer Cetus Instruments, Norwalk, CT) with 2.5 mM $MgCl_2$ was incubated at 42°C for 1 hr followed by 95°C for 2 min and 3 cycles at 94°C for 1 min, 42°C for 1 min and 72°C for 1 min, and subsequently 40 cycles at 94°C for 1 min, 46°C for 1 min and 72°C for 1 min. After exhaustion of the polymerase for 10 min at 72°C, the samples were kept at 4°C.

Amplified products were analyzed by electrophoresis in 2% agarose gels in Tris-acetate buffer and stained with ethidium bromide, and gel purified using a kit (Jetsorb; Genomed, Oeynhausen, Germany) as described by the manufacturers. Dideoxynucleotide sequencing analysis [Sanger et al., 1977] of the PCR-generated 425 base-pair fragment was carried out using a kit from Perkin Elmer (FS polymerase) for automated dyedeoxy cycle sequencing according to the manufacturer's instructions, and analyzed on a ABI 310 automatic sequencing apparatus (Perkin Elmer).

Analysis of nucleotide and deduced amino acid sequence differences among the TBE viruses and alignment of these sequences were performed using the PILEUP program of the GCG software package. Maximum parsimony analysis of the nucleotide sequences were performed using PAUP 4.0.0d55 for Unix [Swofford, 1991]. Phylogenetic trees were calculated using the heuristic search option, and bootstrap confidence limits were obtained from 5,000 heuristic search replicates.

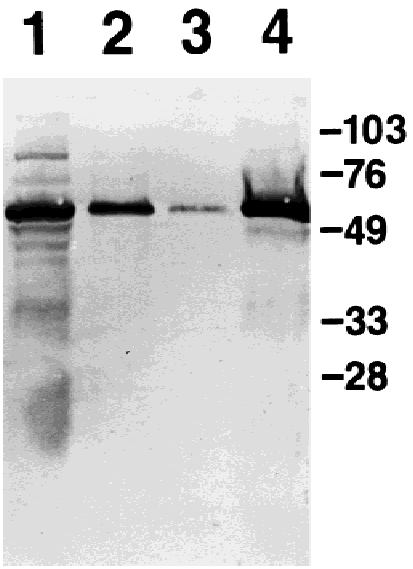

Fig. 1. Pooled brain suspensions from suckling mice inoculated with serum #3684-I were analyzed by immunoblotting. TBEV antigen was detected by a cocktail of 9 Mabs specific for the E protein. The pooled brain suspension was diluted 1:5 **(lane 1),** 1:50 **(lane 2),** 1:500 **(lane 3)**. **Lane 4** represents a cell extract of TBEV strain Hochosterwitz infected chicken fibroblasts diluted 1:10. Molecular weights are indicated on the right.

Virulence in Mice

TBEV strain Hochosterwitz was used to compare the virulence of strain Latvia-1-96 in white mice. Groups of 10 mice were inoculated intraperitoneally with mouse brain suspensions (suckling mouse brain passages 4 and 2 for Hochosterwitz and Latvia-1-96, respectively) containing 1,000 fluorescent focus doses (FFD) of each virus. The mice were examined daily for up to two weeks and mortality and mean survival rates were calculated.

RESULTS

Patient Data

Serum samples from patients with acute clinical symptoms consistent with the first phase of verified TBE (fever, headache) were included in the study. Patient #3684, from which a virus could be isolated, showed typical clinical symptoms. A tick bite, obtained in the Aluksne region (Eastern Latvia), was recognized five days prior to onset of the symptoms. The first serum sample, drawn on the day of onset of symptoms, was TBEV IgM negative, while the second serum sample (day 10) showed seroconversion of TBEV IgM as determined by ELISA.

Virus Isolation

Groups of nine suckling mice were inoculated intracerebrally with acute phase sera from three patients with symptoms compatible with acute TBE (see above). All mice in one of the groups (inoculated with #3684-I) showed signs of infection at day seven and were killed. The other animals remained healthy for 14 days.

TABLE I. IFA Reactivity to Latvia-1-96 (Vero Cell Cultured)

Serum	Reciprocal IFA end-point titer
Patient serum #3684-I (0 d)[a]	<25
Patient serum #3684-II (10 d)	50
TBE late convalescent Swedish human serum	800
Rabbit anti-TBE serum	400
Hamster anti-TBE (Sofjyn #C) serum	100
Human negative control serum	<25
Rabbit negative control serum	<25
Hamster negative control serum	<25

[a]Days after onset of disease within brackets.

Mouse brain suspension was examined for TBE virus antigen by a cocktail of TBEV E protein-specific Mabs by immunoblotting. The result indicated the presence of large amounts of TBE E protein (Fig.1).

Virus containing mouse brain suspension was inoculated on Vero cells, passaged once, and analyzed by IFA. A convalescent phase serum sample from patient #3684 and late convalescent serum from a Swedish patient as well as animal TBE antisera had significant reactivities to the isolated virus (Table I).

Antigenic Characterization

The antigenic characteristics of the isolate was examined by a panel of nine mouse Mabs generated against the TBE strain K23 (western European subtype) and reactive with three antigenic regions of the E protein [Niedrig et al., 1994]. The data revealed similar patterns between the Latvia-1-96 strain, the western European subtype (Hochosterwitz) and the prototype strain of the far eastern subtype (Sofjyn; Table II).

Cross-Neutralization

A RFFIT comparison with antisera from bank voles and hamsters infected experimentally showed similar neutralizing titers to the WTBE (Hochosterwitz), Latvia-1-96, and the FETBE prototype strain (Sofjyn), although in several cases four-fold higher titers to the homologous strain was observed (Table III). Thus, the data indicated Latvia-1-96 to represent a third subtype within the TBE complex.

Sequence Analysis

Sequence analysis of the PCR generated fragment of Latvia-1-96 revealed a significantly higher homology to the Novo-Siberian TBEV strain Vasilchenko, than to the western European strains (Neudoerfl and Kumlinge) or the far eastern strain Sofjyn (Table IV). The nucleotide sequence homology to strain Vasilchenko was as high as 93.7%, while the homologies to the western and far eastern TBE strains ranged from 84.0%–84.6%, respectively. The lowest nucleotide sequence homology was observed to Louping ill virus. The deduced amino acid sequence of the isolate showed a high level of homology between all compared strains, highest to the Vasilchenko and Sofjyn strains.

TABLE II. Mab Reactivity With TBE Strains in IFA

	Virus strains		
Monoclonal antibody	Hochosterwitz	Latvia-1-96	Sofjyn
TBE E Mabs			
694	3200[a]	3200	3200
75	3200	1600	3200
1786	3200	3200	1600
1493	800	400	400
1418	200	400	200
1367	200	400	200
1718	400	800	100
324	25	25	50
171	800	400	100
Neg. Mab			
593	<25	<25	<25

[a]Reciprocal end-point titer.

TABLE III. Cross-Neutralization Data

	Virus (Vero cultured)		
Sera	Hochosterwitz	Latvia-1-96	Sofjyn
Hochosterwitz (bank vole)			
1	≥640[a]	160	160
2	320	160	320
3	320	160	160
Latvia-1-96 (bank vole)			
1	40	320	≥640
2	320	640	320
3	160	640	≥640
Sofjyn			
1 (bank vole)	160	80	320
A (hamster)	80	80	≥320
C (hamster)	40	80	≥320
Neg. hamster			
1	<20	<20	<20
2	<20	<20	<20

[a]Reciprocal end-point titers.

Phylogenetic analysis of the partial E-gene (Fig. 2) showed that the TBE complex, including TBE, Louping ill and Kyasanur Forest Disease viruses, belonged to the same clade, separated from Langat and yellow fever viruses (bootstrap probability of 69%). The TBE strains formed four distinct sub-branches, including the two subtypes of TBE; FETBV and WTBE described previously. Two additional sub-branches were well separated from each other and from the Louping ill virus group and Omsk hemmorrhagic fever virus, with bootstrap probabilities of more than 98%. Latvia-1-96 was shown to group together with the Vasilchenko strain from Siberia, with a bootstrap probability of 99%, thus forming a separate subtype. In the WTBE group, the Swedish strain 3382 of human origin [Haglund et al., unpublished], was found to be separate from the Finnish and Austrian strains Kumlinge and Neudoerfl (bootstrap probability 83%).

Analysis of Mouse Neurovirulence of Latvia-1-96

TBEV strains Hochosterwitz and Latvia-1-96 were compared in a mouse neurovirulence assay by inoculating an estimated 1,000 FFD of virus intraperitoneal-

TABLE IV. Nucleotide (nt 885-1310) and Deduced Amino Acid Identities (%) of Latvia-1-96 E-Gene as Compared to Strains of the TBE Complex

	Latvia	Vasilchenko	Sofjyn	Turkey	Kumlinge	Neudoerfl	Louping Ill
Latvia	xxxx	93.73[a]	84.63	84.32	84.01	84.01	82.75
Vasilchenko	99.05	xxxx	86.52	84.95	84.63	84.63	84.32
Sofjyn	99.05	100	xxxx	84.32	86.83	86.52	84.95
Turkey	96.22	95.28	95.28	xxxx	83.07	82.75	85.57
Kumlinge	97.16	98.11	98.11	97.16	xxxx	99.05	88.08
Neudoerfl	97.16	98.11	98.11	97.16	100	xxxx	87.14
Louping ill	93.39	93.39	93.39	94.33	94.33	94.33	xxxx

[a]Nucleotide identities above, right and deduced amino acid identities down, left.

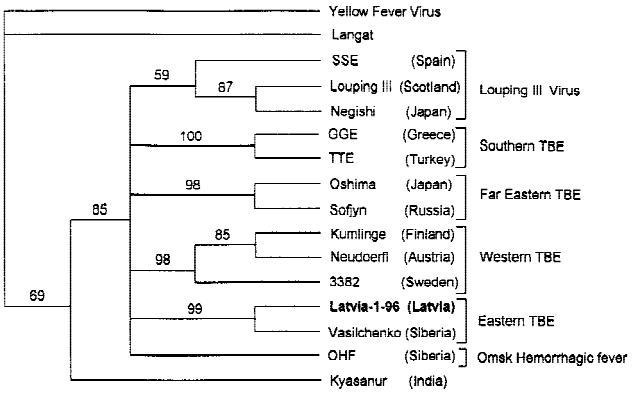

Fig. 2. Phylogenetic relationships of Latvia-1-96 and other TBE- and flaviviruses, based on maximum parsimony analysis of a 319 nt region of the E-gene (nt 952-1270). Bootstrap confidence limits (shown above each interior branch) were calculated from 5000 heuristic search replicates. The geographical origins of the strains are indicated in brackets. Yellow fever virus, strain 17D (Gene Bank accession number U17066); Langat, TP21 (M73835); SSE, Spanish sheep encephalitis (X77470); Louping ill, LI/A (D12936); Negishi (M94956); GGE, Greek goat encephalitis (X77732); TTE, Turkish tick-borne encephalitis (L01265); Oshima, Oshima 5-10 (AB 001026); Sofjyn (X03870); Kumlinge (X60286); Neudoerfl (U27495); 3382, Swedish TBE-strain (Haglund et al, unpublished); Latvia-1-96 (AJ010192); Vasilchenko (M97369); OHF, Omsk hemorrhagic fever (X66694); Kyasanur, Kyasanur forest disease (X74111).

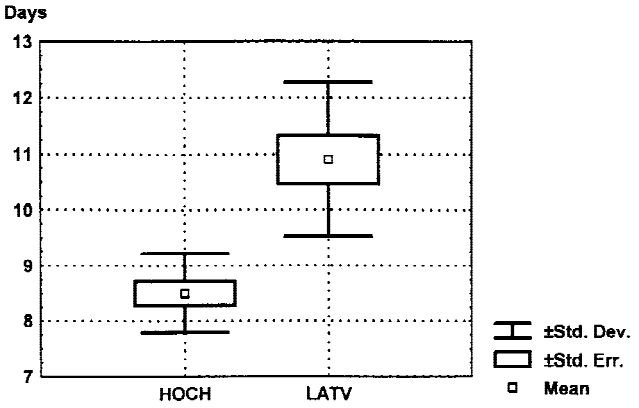

Fig. 3. Mean survival rates **(small boxes)** of mice inoculated with Hochosterwitz (HOCH) or Latvia-1-96 (LATV). The **large boxes** indicate the standard error and the horizontal bars the standard deviation, respectively.

ly into 3-week-old female white mice. None of the mice survived inoculation of either strain Hochosterwitz or Latvia-1-96. However, the average survival rates were 8.5 ± 0.67 days for mice inoculated with Hochosterwitz, in contrast to 10.9 ± 1.3 days for the group inoculated with Latvia-1-96 ($P < 0.001$, t-test for independent samples; Fig. 3).

DISCUSSION

This study describes the isolation and initial characterisation of TBE virus from Latvia. TBE constitutes a significant health problem in Latvia with up to a 1,000 cases confirmed serologically each year [Kalnina et al., 1997]. The knowledge regarding TBE virus in Latvia is limited and there are no reports on patient isolates, and no reports on seroepidemiological or clinical data on TBE in Latvia have been published to date.

The panel of TBEV E-specific monoclonal antibodies [Niedrig et al., 1994] clearly identified the patient isolate as TBEV. This panel proved to be a most convenient tool for initial identification as shown by the highly sensitive detection directly in mouse brain suspensions (by immunoblotting) and for the cell cultured strain (by immunofluorescence assay).

The isolate Latvia-1-96 was distinguished from the prototype strains of WTBEV and FETBEV. Significant (at least four-fold) differences between homologous and heterologous neutralizing titers were observed in both directions when compared to WTBEV, while the comparison to FETBEV revealed four-fold difference in one direction only. Thus, the serological characterization indicated that Latvia-1-96 may represent a third subtype of TBEV.

The sequence similarities were in agreement with the serological data. The nucleotide sequence similarity to the Siberian Vasilchenko strain was significantly higher than the homologies to the WTBEV and FETBEV prototype strains. The overall higher sequence homologies for the deduced amino acids were in line with the previously reported high degree of sequence conservation within this region of the E protein [Heinz et al., 1990; Gritsun et al., 1993]. The phylogenetic analysis supported the close relationship of Latvia-1-96 and the Vasilchenko strain; these two strains were situated together in a separate sub-branch with high (99%) bootstrap probability. In addition, the phylogenetic data suggested the TBE-like strains from Greece and Turkey to represent a fourth subtype of TBEV [Marin et al., 1995; Whitby et al., 1993b].

These findings were to some extent unexpected. Previous studies have revealed an extremely high degree

of sequence homology between TBEV strains from different geographical areas in central and northern Europe, e.g. Kumlinge (Finland), Neudoerfl (Austria) and strains from Sweden [Whitby et al., 1993a] [Haglund et al., unpublished]. Accordingly, strains of the far eastern subtype (e.g. Sofjyn and Oshima) are most similar [Takashima et al., 1997]. WTBE has *I. ricinus* as the main vector, while the FETBEV strains have another tick species, *I. persulcatus*, as the main vector. Both *I. ricinus* and *I. persulcatus* are present in Latvia; *I. ricinus* mainly in the western part and *I. persulcatus* mainly in the eastern part of the country. Therefore, the expected TBE virus variant in Latvia may have been either of the western or eastern subtypes. It should be noted that the patient contracted a tick bite in the eastern part of Latvia (Aluksne region) and it is thereby likely that Latvia-1-96-like TBEV strains are carried by *I. persulcatus*. To date, only one Latvian TBEV strain, RK1424 isolated from *I. persulcatus* in 1977, has been described in the literature [Holzmann et al., 1992; Wallner et al., 1995]. Although the characterization of this strain is still limited, antigenic comparison indicated RK1424 to be most similar to the far eastern prototype strain Sofjyn [Holzmann et al., 1992]. Also the western variant of TBEV carried by *I. ricinus* may be circulating in Latvia, as serologically confirmed TBE occurs all over the country, including regions with *I. ricinus* predominance.

Our data on virulence in mice revealed a significant difference between WTBEV and Latvia-1-96. This is in agreement with the low virulence of the Vasilchenko strain reported previously [Asher, 1979; Frolova et al., 1982] and also with the relatively mild clinical course of the patient from which the virus originated. If more than one variant of TBEV circulates in Latvia, i.e. also typical WTBEV, it is possible that the more severe forms of clinical disease may be caused by this variant. Our initial trials to associate disease severity with specific geographical areas of Latvia have so far been unsuccessful. In order to evaluate fully and examine a possible connection between an infecting virus variant and the clinical course in a patient, more accurate and standardized criteria for the description of clinical symptoms as well as characterization of further human isolates are needed.

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